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COMMUNICATION

CCAAT/enhancer binding proteins play a role in *ori*Lyt-dependent genome replication during MHV-68 *de novo* infection

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ABSTRACT

Murine gammaherpesvirus 68 (MHV-68), a member of the gammaherpesvirus family, replicates robustly in permissive cell lines and is able to infect laboratory mice. MHV-68 has emerged as a model for studying the basic aspects of viral replication and host–virus interactions of its human counterparts. Herpesvirus genome replication is mediated through a *cis*-element in the viral genome called the origin of lytic replication (*ori*Lyt). A family of transcription factors, CCAAT/enhancer binding proteins (C/EBPs), assists in *ori*Lyt-mediated DNA replication during gammaherpesvirus reactivation. In this study, we examined the role of C/EBPs in gammaherpesvirus DNA replication during *de novo* infection, using MHV-68 as a model. We found that C/EBP α and β bind to the CCAAT boxes in the MHV-68 *ori*Lyt core region both *in vitro* and *in vivo*, as demonstrated by electrophoretic mobility shift assay and chromatin immunoprecipitation assay. A dominant negative form of C/EBPs significantly impaired the lytic replication efficiency of MHV-68 on both the plasmid and genome levels in a replication assay, indicating that functional C/EBPs are required for maximal MHV-68 genome DNA replication. Collectively, our data demonstrate that C/EBPs interact with the *ori*Lyt core region and play an important role in MHV-68 lytic DNA replication during *de novo* infection.

KEYWORDS C/EBPs, murine gammaherpesvirus 68, *ori*Lyt, lytic replication

INTRODUCTION

Human gammaherpesviruses, Kaposi's sarcoma-associated herpesvirus (KSHV), and Epstein-Barr virus (EBV) are closely linked to malignant tumors (Kieff and Rickinson, 2001; Moore and Chang, 2001). However, studies of KSHV and EBV are relatively limited due to their restricted host ranges and lack of permissive cell lines that can efficiently support their proliferation. Murine gammaherpesvirus 68 (MHV68) also belongs to the gamma herpesvirus subfamily and shares many similarities with KSHV and EBV, both genetically and biologically (Efstathiou et al., 1990; Virgin et al., 1997). MHV-68 replicates robustly in permissive cell lines and produces progeny viruses with a high titer. MHV-68 infection of mice also provides a tractable small animal model for further investigation of the mechanisms of viral infection and the pathogenesis of gammaherpesviruses (Simas and Efstathiou, 1998).

Like all other herpesviruses, gammaherpesviruses have two life cycles: latency and lytic replication (Roizman and Pellett, 2001b). During latency, the viral genome replicates synchronously with the host genome and is maintained in host cells in an extrachromosomal manner (Collins et al., 2002; Hu and Renne, 2005). During the lytic cycle, the virally encoded DNA replication proteins gather at the origin of lytic replication (*ori*Lyt) to initiate the replication process, and the newly synthesized viral DNAs are processed at terminal repeats and packaged into progeny virions (Kieff and Rickinson, 2001; Mocarski and Courcelle, 2001; Roizman and Knipe, 2001a).

Previous studies on gammaherpesviruses reveal that both cellular and viral proteins are involved in lytic viral DNA replication. A family of transcription factors, CCAAT/enhancer

binding proteins (C/EBPs), assists in the lytic genome replication of EBV and KSHV during viral reactivation (Wu et al., 2003; Wang et al., 2004; Huang et al., 2006). The C/EBP family is composed of six members (C/EBPα–C/EBPζ), which all contain conserved basic-leucine zipper (bZIP) domains at their C-terminus and an activation domain at their N-terminus. Via their C-terminal DNA binding domain, they interact with DNA sequence especially CCAAT motifs in promoter regions to modulate gene expression (Landschulz et al., 1988; Ramji and Foka, 2002). Research on EBV and KSHV has shown that C/EBPs play a role in viral DNA replication during reactivation through interaction with the core region of the EBV and KSHV *ori*Lyts (Wu et al., 2003; Wang et al., 2004; Huang et al., 2006).

Our group has identified two *ori*Lyts in the MHV-68 genome, which are located toward the right (nt. 100,724–101,975) and left (nt. 25,695–26,883) ends of the genome (termed the right and left *ori*Lyt, respectively) (Deng et al., 2004; Gong et al., 2009). The two *ori*Lyts of MHV-68 share several conserved *cis*-elements, including two pairs of CCAAT boxes, an AT-rich palindrome, and a GC-rich repeat region. We further analyzed the left *ori*Lyt in detail through deletion mutation and site-directed mutagenesis and found that the CCAAT boxes are indispensable for the function of the left *ori*Lyt (Deng et al., 2004; Gong et al., 2009). In the current study, we aimed to test whether C/EBPs bind to the core region of MHV-68 *ori*Lyt and, if so, whether such interactions play a functional role in mediating MHV-68 lytic genome replication.

RESULTS AND DISCUSSION

C/EBPs bind to MHV-68 *ori*Lyts *in vitro*

We first used electrophoretic mobility shift assays (EMSAs) to test whether C/EBPs bind to the core region of MHV-68 *ori*Lyts. Oligonucleotides were synthesized for EMSA as follows: L1 + 2 covered CCAAT boxes 1 and 2 present in the MHV-68 left *ori*Lyt; L3 + 4 covered CCAAT boxes 3 and 4; R1 + 2 and R3 + 4 were designed in the same manner based on the right *ori*Lyt (Fig. 1A). As controls, Ori-L1 and Ori-L3 were synthesized according to a previous study showing that C/EBPs bind to fragments containing paired CCAAT boxes in the KSHV *ori*Lyt (Wu et al., 2003). We transfected 293T cells with expression plasmids for either FLAG-tagged C/EBPα (pHC125B) or C/EBPβ (pHC108C), and prepared nuclear extracts for EMSAs. Our result demonstrated that C/EBPα and β bind to Ori-L1 and Ori-L3, as well as the C/EBP consensus sequence (arrowhead, Fig. 1B, lanes 1, 10 and 13; Fig. 1C, lanes 1, 10 and 13). A complex with similar mobility was observed when oligonucleotides L1 + 2 and L3 + 4 were used (arrowhead, Fig. 1B, lanes 4 and 7; Fig. 1C, lanes 4 and 7), indicating that C/EBPs are able to bind to the core region of the MHV-68 left *ori*Lyt. To confirm the specificity of C/EBP binding, we conducted supershift and competition experiments. Addition of an antibody against the FLAG epitope successfully supershifted the complex (arrow, Fig. 1B, lanes 2, 5, 8, 11 and 14; Fig. 1C, lanes 2, 5, 8, 11 and 14), suggesting that FLAG-tagged C/EBPs are indeed part of the supershifted complex. Moreover, the intensity of the shifted bands decreased in the presence of unlabeled C/EBP consensus oligos (Fig. 1B, lanes 3, 6, 9, 12 and 15; Fig. 1C lanes 3, 6, 9, 12 and 15), further proving that the interaction between C/EBPs and L1 + 2 or L3 + 4 are specific. Likewise, C/EBPα and β also bound to the core region of the MHV-68 right *ori*Lyt, though with lower efficiency (Fig. 2C, lanes 3, 4, 9 and 10; and Fig. 2D, lanes 3, 4, 9 and 10). It should be noted that in addition to this complex, other bands were also detected with probe L1 + 2 or L3 + 4. This was presumably caused by non-specific binding of the probe with the nuclear extract because these bands were neither supershifted nor inhibited by competitors.

The observation that C/EBPα and β bound to the MHV-68 left and right *ori*Lyt core regions is in agreement with previous studies of KSHV and EBV (Wu et al., 2003; Huang et al., 2006). In KSHV, the left and right *ori*Lyts share an almost identical 1.15-kb sequence organized in opposite directions. A previous study demonstrated that each KSHV *ori*Lyt harbors three C/EBP binding regions, as detected by probes Ori-L1, Ori-L3, and Ori-V in EMSAs. However, the specific shifted bands representing C/EBP-DNA complexes were somewhat smeared, and non-specific shifts were also detected (Wu et al., 2003). For comparison, we included Ori-L1 and Ori-L3 in our EMSAs. Our results demonstrated that C/EBPs bound to the CCAAT boxes in the MHV-68 left *ori*Lyt region (L1 + 2 and L3 + 4) with similar efficiency to KSHV Ori-L3 but with higher efficiency than KSHV Ori-L1, although non-specific bands were also detected (Fig. 1B and 1C, lanes 4, 7, 10 and 13).

C/EBPs bind to *ori*Lyts in the MHV-68 genome during *de novo* infection

To examine whether C/EBPs bind to the MHV-68 *ori*Lyt core region of the viral genome *in vivo*, chromatin immunoprecipitation (ChIP) assays were performed. Cells transfected with FLAG-tagged C/EBPα or β were infected with MHV-68, and DNA-protein complexes were cross-linked by formaldehyde and used for ChIP assays. The resulting DNA fragments were amplified with specific primers toward the MHV-68 left or right *ori*Lyt core region. PCR products of the expected length were detected when the anti-FLAG antibody was used for immunoprecipitation (Fig. 3, top 2 panels, lanes 3 and 6) but not when control IgG was used (Fig. 3, top 2 panels, lanes 2 and 5). Moreover, PCR reactions using primers specific for the cellular beta-actin coding sequence or the intron region of the MHV-68 *rta* gene, which do not contain C/EBP binding sites, yielded no products (Fig. 3, bottom 2 panels, lanes 2, 3,

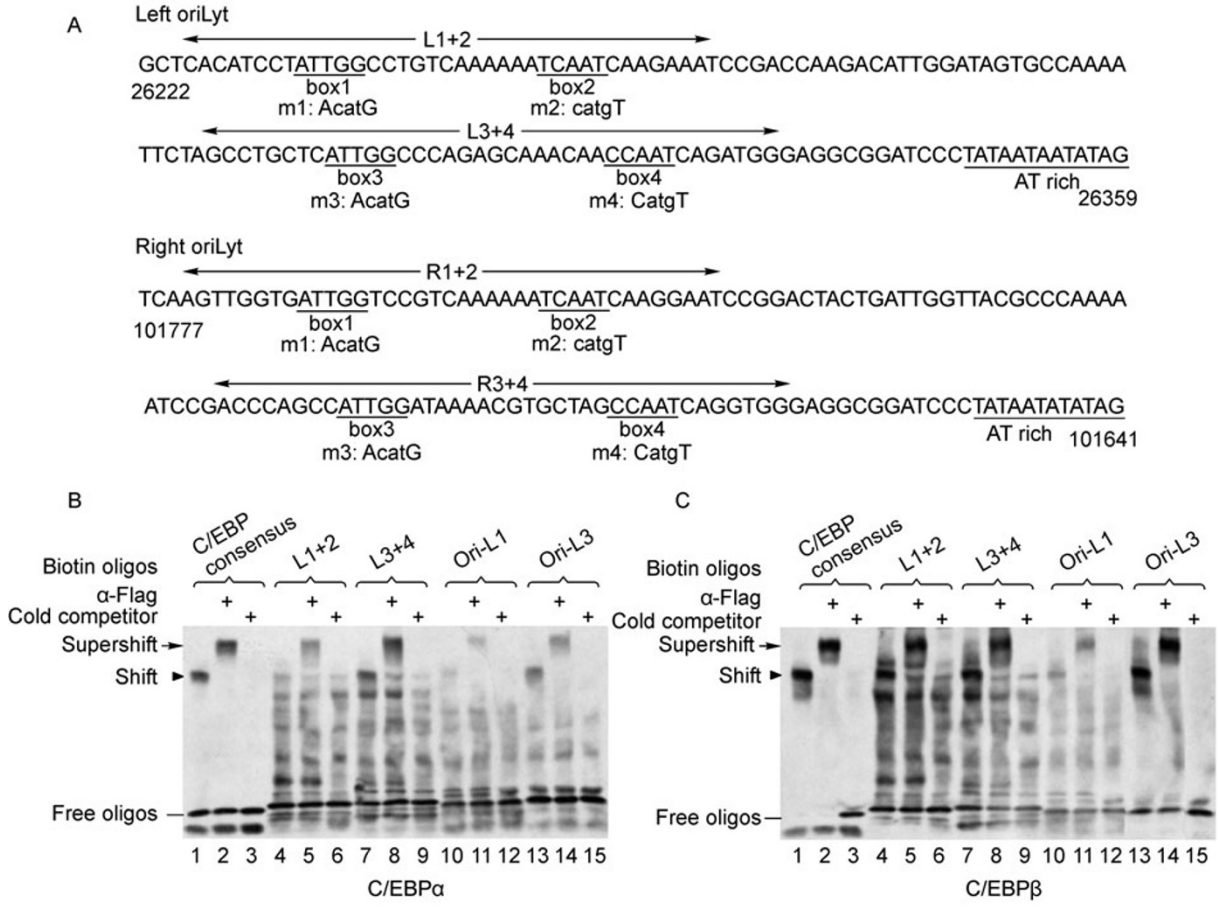

Figure 1. C/EBPα and β bind to MHV-68 *ori*Lyt core region *in vitro*. (A) Sequence of the MHV-68 left and right *ori*Lyt core regions and schematic diagram of the probes used in EMSA assay. Mutations engineered in each of the CCAAT boxes were indicated with lower case letters beneath the wild type sequences. C/EBPα (B) or C/EBPβ (C) binds to MHV-68 left *ori*Lyt in EMSA. Anti-FLAG antibody was employed to supershift the C/EBP-DNA complex, and 100-fold excess unlabeled C/EBP consensus oligonucleotides was used as cold competitor. Biotin-labeled C/EBP consensus oligonucleotides were used as positive control for C/EBP binding.

5 and 6). These results demonstrate that both C/EBPα and β are able to bind to the *ori*Lyt core region in the MHV-68 genome during *de novo* infection.

Two CCAAT boxes in the MHV-68 left *ori*Lyt are necessary for C/EBP binding

Previous functional studies demonstrate that CCAAT boxes are crucial *cis*-elements for *ori*Lyt-dependent DNA replication during KSHV reactivation (Wang et al., 2004). By EMSAs, the second CCAAT box of Ori-L1 is essential for C/EBP binding, whereas the first CCAAT box is dispensable (Wu et al., 2003). Our group revealed that CCAAT boxes are essential *cis*-elements for the function of the MHV-68 left *ori*Lyt (Gong et al., 2009). To investigate whether the CCAAT boxes in the MHV-68 *ori*Lyt core region are also important for C/EBP binding, we designed eight oligonucleotides based on L1 + 2, L3 + 4, R1 + 2, and R3 + 4; each of them contained mutations in one CCAAT box (Fig. 1A). The oligonucleotides were biotin-labeled and used for EMSAs as described above. Anti-FLAG antibody was again introduced to confirm the specificity of binding. Our results demonstrate that both C/EBPα and C/EBPβ were able to bind to L1 + m2 and L3 + m4 with similar affinity compared to wild type sequences, whereas the binding affinities to Lm1 + 2 and Lm3 + 4 were much reduced, suggesting that CCAAT box 1 and especially box 3 are important for mediating binding of C/EBPs to the left *ori*Lyt (Fig. 2A and 2B). Intriguingly, the CCAAT boxes in the right *ori*Lyt were largely dispensable for C/EBPα or β binding when compared to those from the left *ori*Lyt (Fig. 2C and 2D). Together, these results suggest that CCAAT boxes 1 and 3 from the left *ori*Lyt play a pivotal role in the binding of C/EBPs *in vitro*.

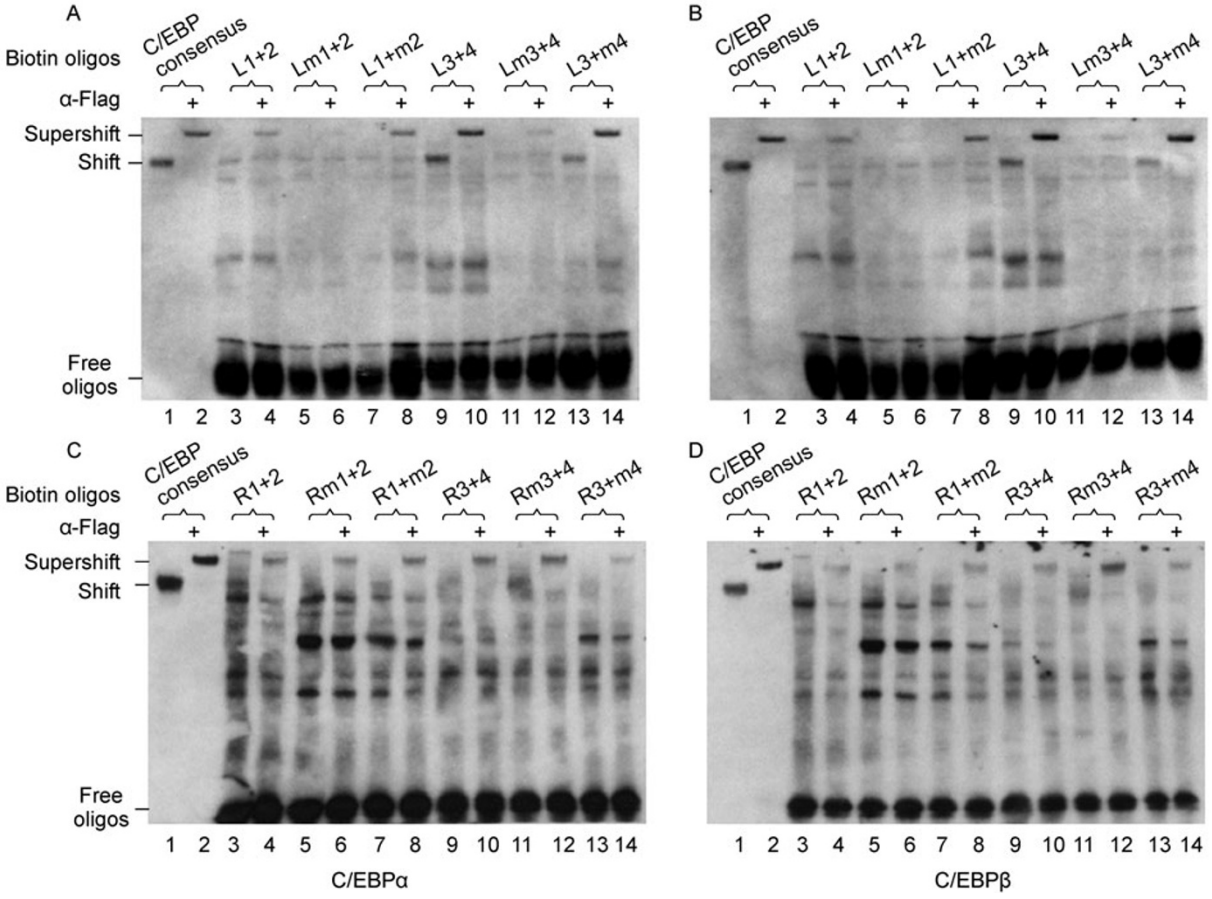

Figure 2. Characterization of the specific binding sequence for C/EBP proteins in MHV-68 *ori*Lyt regions by EMSA. Nuclear extract was harvested from 293T cells transfected with FLAG-tagged C/EBPα or C/EBPβ. Oligonucleotides containing mutated CCAAT boxes, as illustrated in Fig. 1A, were biotin-labeled and applied for EMSA to test their binding efficiency by C/EBPα (A and C) and β (B and D) proteins.

Functional C/EBPs are required for maximal *ori*Lyt-mediated lytic DNA replication during MHV-68 *de novo* infection

Given the fact that C/EBPs bind to CCAAT boxes in the MHV-68 *ori*Lyt, we next conducted a knock-down experiment to evaluate the functional significance of such interactions during *de novo* MHV-68 infection. CHOP10 is a C/EBP family member that heterodimerizes with C/EBPα or β and impairs their DNA binding ability, consequently serving as a dominant negative mutant of C/EBPs. We first cloned the CHOP10 sequence into pCMV-HA to generate pCMVHA-hCHOP10 and confirmed the expression of hCHOP10 by western blotting (data not shown). We then co-transfected pMOL (bearing the 1.1-kb MHV-68 left *ori*Lyt) (Gong et al., 2009) and pCMVHA-hCHOP10 (or pCMV-HA as a vector control) into 293T cells. Twenty four hours later, we infected cells with MHV-68 to provide trans-factors required for DNA replication and prepared total cellular DNA for Southern blotting. The replication efficiency of pMOL was significantly impaired by CHOP10 (Fig. 4A, lanes 2, 4 and 6) compared to controls (Fig. 4A, lanes 1, 3 and 5), suggesting that C/EBPs play important roles in *ori*Lyt-mediated DNA replication in this plasmid system.

To further explore the contribution of C/EBPs in mediating MHV-68 lytic genome replication during *de novo* infection, we transfected 293T cells with pCMVHA-hCHOP10 or pCMV-HA and then infected them with MHV-68. Total cellular DNAs were harvested, and the replication of the MHV-68 genome was analyzed. Southern blotting demonstrated that the replication efficiency of the MHV-68 genome decreased by ~50% in the presence of hCHOP10 (Fig. 4B), indicating that a functional C/EBP-DNA complex is required for maximal MHV-68 lytic genome replication. The reason that the decrease of genome replication efficiency was not as large as in the plasmid replication assay may be two-fold. First, pCMV-hCHOP10 may not have been transfected into and expressed in every cell that was later infected with MHV-68. Second,

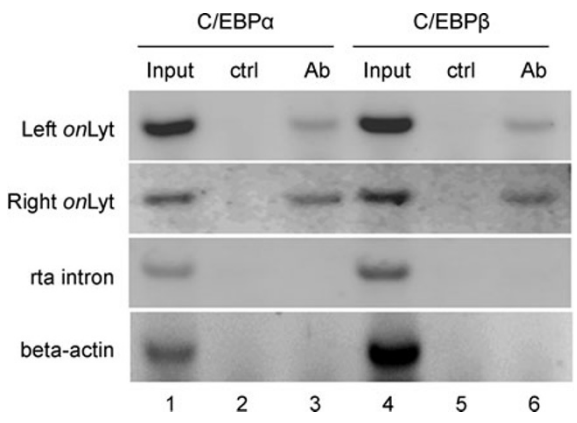

Figure 3. C/EBPα and β interact with the *ori*Lyt regions in the MHV-68 genome *in vivo*. 293T cells were transfected with FLAG-tagged C/EBPα or C/EBPβ followed by infection with MHV-68. DNA-protein complexes were cross-linked at 12 h post-infection. Sheared DNA-protein complexes were precipitated with a monoclonal antibody against FLAG epitope, and the precipitated DNA was amplified by PCR with primers specific for the *ori*Lyt regions, MHV-68 *rta* gene intron or beta-actin coding region. Immunoprecipitation using an isotype-matched mouse IgG antibody served as negative controls.

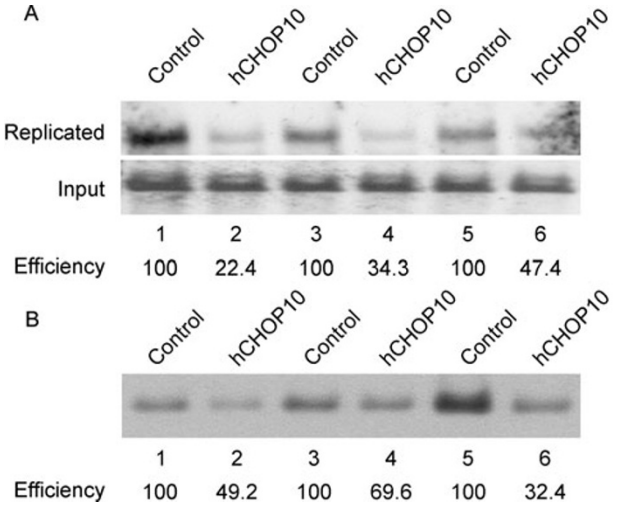

Figure 4. A dominant negative form of C/EBP decreased the efficiency of *ori*Lyt-dependent MHV-68 DNA replication. (A) CHOP10 significantly impaired the replication efficiency of pMOL. 293T cells were co-transfected with pMOL and pCMVHA-hCHOP10 (or pCMVHA as a vector control), followed by infection with MHV-68. Southern blotting was performed with DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) using a probe against the pGEM-T vector. Lanes 1 to 6 represent samples from three independent transfection experiments. Replication efficiencies were quantified and presented, and the efficiency from samples transfected with the vector control was set as 100. (B) The replication efficiency of MHV-68 genome was attenuated by CHOP10. 293T cells were transfected with pCMVHA-hCHOP10 or its vector, infected by wild type MHV-68 and harvested for Southern blotting. *Not*I was used to digest genomic DNA and a probe against the MHV-68 terminal repeat region was employed. Three independent experiments were presented.

although *Dpn*I digestion can be utilized to eliminate the input plasmid DNA before Southern blotting, as done in the plasmid assay system (Fig. 4A), such manipulation is futile for viral genomes. Hence, the newly synthesized viral genomes and the input parental genomes cannot be discriminated by Southern blotting, masking the dominant negative effect of CHOP10 to certain extent (Fig. 4B).

In the present study, we demonstrated that C/EBPα and β interact with the MHV-68 *ori*Lyt regions, both *in vitro* and *in vivo*, via binding to the CCAAT boxes. By introducing mutations into the CCAAT boxes, we found that CCAAT boxes 1 and 3 in the left *ori*Lyt are crucial for C/EBP binding. Moreover, a "dominant negative C/EBP mutation" (i.e. CHOP10 expression) remarkably inhibited the lytic replication of MHV-68, both in a plasmid system and on the genome level, suggesting that functional C/EBPs are important for left *ori*Lyt-mediated DNA replication of MHV-68 during *de novo* infection. In comparison, although the left and right KSHV *ori*Lyts identified in a plasmid system share an almost identical 1.15-kb region, Xu et al. reported that on the genome level, the left *ori*Lyt is sufficient to replicate the KSHV genome, whereas the right *ori*Lyt alone fails to propagate the viral genome, indicating that functional variation may exist between the left and right *ori*Lyt regions of gammaherpesviruses. Consistent with these results, we found that although both the left and right *ori*Lyt regions of the MHV-68 genome contain CCAAT boxes, only boxes 1 and 3 in the left *ori*Lyt are important for mediating C/EBPs binding.

Most studies of the lytic replication of EBV or KSHV have been performed using latently-infected cell lines due to the lack of a permissive infection system; little is known about the *de novo* infection process. Therefore, although C/EBPs are reported to contribute to the lytic replication of KSHV and EBV during reactivation (Wu et al., 2003; Wang et al., 2004; Huang et al., 2006), it remains to be determined whether such a scenario also takes place during *de novo* infection. Fortunately, the robust replication of MHV-68 in tissue culture enabled us to evaluate the role of C/EBPs during *de novo* infection for the first time and helps in a more comprehensive understanding of gammaherpesvirus lytic replication.

Nevertheless, because the mechanism of viral lytic replication is complicated, multiple trans-factors, both viral and cellular, may be involved. We previously reported that a ubiquitous cellular transcription factor, NF-Y, binds to CCAAT boxes 1, 3, and 4 in the MHV-68 left *ori*Lyt, both *in vitro* and *in vivo*, and plays an important role in mediating lytic viral genome replication during *de novo* infection (Gong et al.,

2009). Together with our current findings, we hypothesize that MHV-68 may utilize NF-Y to pre-set the architecture of the *ori*Lyt and then employ C/EBPs to proceed with DNA replication because NF-Y and C/EBP can interact with each other (Xu et al., 2006). It is also possible that NF-Y and C/EBP bind to the *ori*Lyt in a simultaneous manner and facilitate efficient lytic DNA replication. To dissect this, future investigations of the detailed mechanisms governing C/EBP's and NF-Y's role in MHV-68 lytic genome replication are required.

MATERIALS AND METHODS

Cell culture and viruses

BHK-21 and 293T cells were maintained in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (Hyclone) and antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin) at 37°C in the presence of 5% CO_2. MHV-68 was propagated by infecting BHK-21 cells at a multiplicity of infection (MOI) of 0.05 plaque forming units (PFU)/cell. Viral titers were determined by standard plaque assays.

Plasmids

The pMOL plasmid was constructed by cloning a 1.2-kb fragment (NC_001826, nt. 25,695–26,883, amplified by PCR) of MHV-68 DNA into the pGEM-T vector (Promega), as described previously (Gong et al., 2009). To generate plasmid pCMV-hCHOP10, human CHOP10 was amplified by PCR from 293T cell cDNA and cloned into pCMV-HA (Clontech). The FLAG-tagged C/EBPα and C/EBPβ expression plasmids, pHC125B and pHC108C, were kindly provided by Dr. S. Diane Hayward (Huang et al., 2006).

EMSAs

293T cells were transfected with 12 μg pHC125B or pHC108C. Forty-eight hours post-transfection, cells were harvested, and nuclear extracts prepared using a modified method described by Schreiber (Schreiber et al., 1989). Oligonucleotides were prepared as described in the Biotin 3′ End DNA Labeling Kit (Pierce). DNA-protein binding reactions and EMSAs were performed according to the LightShift Chemiluminescent EMSA Kit (Pierce). For supershift experiments, 1 μL anti-FLAG antibody (Sigma) was added to DNA-protein binding reactions. For competition assays, 100-fold excess unlabeled C/EBP consensus oligonucleotide was added.

Oligonucleotides used in the EMSAs are as follows: C/EBP consensus, 5′-TGCAGATTGCGCAATCTG-3′; Ori-L1, 5′-CGCTGATTGGTTCCCGCTCTGGGCCAATCA GCA-3′; and Ori-L3, 5′-CCGAGATTGGTCGGCCGGATGGGCCAATGGCGA-3′ (Wu et al., 2003). The sequences of L1 + 2, L3 + 4, Lm1 + 2, L1 + m2, Lm3 + 4, L3 + m4, R1 + 2, R3 + 4, Rm1 + 2, R1 + m2, Rm3 + 4, and R3 + m4 are illustrated in Fig. 1A.

ChIP

Two million 293T cells were transfected with pHC125B or pHC108C, and 24 h later, cells were infected with wild type MHV-68 at a MOI of 5. Twelve hours later, proteins were cross-linked to DNA by adding formaldehyde to a final concentration of 1%, and ChIP assays were performed according to manufacturer's instructions (Upstate). The primer pairs used are as follows: left *ori*Lyt, 5′-GCTATGTTTGAC TTTTCGCTGTTTCG-3′ and 5′-AAGGGGATTTCCAGGTA-GAGGGTCTTC-3′; right *ori*Lyt, 5′-AGGGATCCGCCTCCCACCTG-3′ and 5′-CTCTGCCGCATCGCCTCACA-3′; RTA intron, 5′-TTTTCTCAAGGCTTCCTCGTCT-3′ and 5′-GGC ACTGTCAATT-TACTGGGCT-3′; and human beta-actin coding region, 5′-GGACTT CGAGCAAGAGATGG-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′.

De novo infection-replication assays and Southern blotting

Plasmids were transfected into 293T cells in 6-well plates with jetPEI (Polyplus). Twenty-four hours post-transfection, cells were infected with wild type MHV-68 at a MOI of 0.1. When > 95% cells showed cytopathic effect (CPE) at 72–96 h post-infection, cells were harvested, and total cellular DNA was extracted. One-twelfth of the DNAs were digested overnight with *Dpn*I and *Pst*I to examine newly replicated plasmid DNA (or *Pst*I alone to examine input DNA) and subjected to Southern blotting. For Southern blotting analysis, digested DNAs were separated on a 0.8% agarose gel in 1×TAE buffer. The gel was treated with 0.25 mol/L HCl, followed by alkaline denaturation and neutralization. DNAs were transferred onto a Hybond-N + membrane (Amersham Pharmacia) via capillary transfer in 10×SSC buffer and immobilized by UV-crosslinking. Southern blotting was performed using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) with a probe against the pGEM-T vector.

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ABBREVIATIONS

C/EBPs, CCAAT/enhancer binding proteins; ChIP, chromatin immunoprecipitation; EBV, Epstein-Barr virus; EMSA, electrophoretic mobility shift assay; KSHV, Kaposi's sarcoma-associated herpesvirus; MHV-68, murine gammaherpesvirus 68; MOI, multiplicity of infection; OBP, origin binding protein; *ori*Lyt, origin of lytic replication

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